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Profiles of GBV-C/Hepatitis G Virus Markers in Patients Coinfected With Hepatitis C Virus

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GBV-C/Hepatitis G virus (GBV-C/HGV) is a newly discovered viral agent, found widely among healthy blood donors and among individuals at risk of parenterally transmitted infections. GBV-C/HGV is found frequently in coinfection with HCV. A population of 109 HCV positive patients was examined for the presence of GBV-C/HGV RNA and antibodies to E2. Of the 109 patients, 23 (21%) had serum GBV-C/HGV RNA in serum, 39 (36%) had only antibodies to E2 and 8 (7%) were positive for both markers, with an overall prevalence of 64%. Different serologic and virological patterns were observed in GBV-C/HGV exposed patients according to their infection status. Active infection was characterized by positive RT/PCR signal with primers for both the 5′UTR and NS5 genomic regions, viremia levels above 10^4 copies/mL by real time quantitative RT/PCR and absence of detectable anti-E2. In the transition phase between active infection and recovery, GBV-C/HGV RNA was only detectable by RT/PCR using primers from the 5′ untranslated region and viremia levels were below 10^4 copies/ml by quantitative PCR, with or without simultaneous presence of anti-E2 antibodies. Resolved infection was characterized by absence of detectable viremia and, in most patients, by the presence of anti-E2. ***J. Med. Virol. 59:45–51, 1999.***

KEY WORDS: GBV-C/HGV; anti-E2, RNA; real time PCR

INTRODUCTION

GB virus C (GBV-C) is a viral agent isolated from individuals with idiopathic hepatitis [Simons et al., 1995]. Independently, another isolate of GBV-C, named hepatitis G virus (HGV), was identified in human plasma [Linnen et al., 1996]. GBV-C and HGV molecular analysis shows that they are both isolates from the same viral species and that they are related to the Flaviviridae family, its genomic structure resembling that of hepatitis C virus (HCV) [Alter 1996, Leary et al., 1996 Linnen et al., 1996]. At present, the precise replication site of GBV-C/HGV remains to be established; its hepatotropic nature remains controversial [Madejon et al., 1997 Laskus et al., 1998; Radkowski et al., 1998].

Although GBV-C/HGV was first isolated from serum samples of patients with chronic hepatitis of unknown etiology, it is now evident that GBV-C/HGV is not the main cause of nonA-E hepatitis [Alter M et al., 1997; Alter H et al., 1997]. The clinical relevance of this agent is still unresolved [Miyakawa and Zuckerman, 1997 Mushahwar et al., 1998]. GBV-C/HGV is found commonly among healthy blood donors around the world (1–5% are GBV-C/HGV RNA positive) [Linnen et al., 1996] and it is particularly prevalent in groups at risk of parenteral exposure [Stark et al., 1996; De Fillippi et al., 1997; Thomas et al., 1997]. GBV-C/HGV is therefore found frequently in coinfection with other parenterally transmitted viral agents, such as HCV [Tanaka et al., 1996]. GBV-C/HGV is sensitive to antiviral therapy, although patients seldom achieve sustained response [Lau et al., 1997; Martinot et al., 1997; McHutchinson et al., 1997; Enomoto et al., 1998].

There are currently two serological markers for GBV-C/HGV exposure. Antibodies to E2 are usually detected in patients who have been in contact with GBV-C/HGV and have cleared the infection. On the other hand, active infection is assessed by isolation of viral RNA in serum samples by RT/PCR of conserved regions, usually 5′UTR, NS5 or NS3 [Schlueter et al., 1996]. Both markers, anti-E2 and RNA, are most often mutually exclusive [Tacke et al., 1997a].

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TABLE I. Epidemiological Variables According to GBV-C/HGV Exposure*

Total patients n = 109	Group 1: RNA+ n = 31	Group 2: RNA− anti-E2+ n = 39	Group 3: non exposed n = 39
Age (years)	36 ± 12[a]	44 ± 15[b]	41 ± 12[c]
Sex (% male)	65%	64%	62%
Tx history	20 (29%)		18 (46%)
IVDA history	30 (43%)[d]		7 (18%)[e]
Age at infection	21 ± 24	19 ± 8	20 ± 12
Duration of infection	14 ± 7[f]	19 ± 10[g]	22 ± 8[h]
ALT (IU/L)	86 ± 84	76 ± 51	69 ± 52
γGT (IU/L)	44 ± 51	49 ± 43	40 ± 28
HAI score	5 ± 4	6 ± 4	6 ± 4
HCV RNA log10	4.0 ± 2.3	4.6 ± 1.8	4.9 ± 1.7
HCV genotype 1	19 (61%)	28 (72%)	31 (79%)
2	1 (3%)	2 (5%)	1 (3%)
3	4 (13%)	1 (3%)	4 (10%)
4	4 (13%)	4 (10%)	1 (3%)

*Tx, transfusion. a vs. b, c $P < 0.002$. d vs. e $P = 0.015$. f vs. g, h $P < 0.002$.

The present study was undertaken to clarify the natural history of GBV-C/HGV infection and to characterize serological and virological patterns of both active and resolved infection in patients with GBV-C/HGV and HCV coinfection.

MATERIALS AND METHODS

Subjects

One hundred nine HCV positive patients were screened for the presence of GBV-C/HGV RNA and antibodies to E2. GBV-C/HGV RNA was quantitated in viremic patients. Twenty-one GBV-C/HGV infected subjects, negative for HCV, HBV, and HIV markers identified previously, were included as quantitation controls. A subset of nine HCV/HGV positive patients was followed up before, during and after IFN treatment. The Ethic Committee of the Institution approved this study.

Serum GBV-C/HGV RNA Screening Procedure

All blood samples were drawn into vacutainer™ tubes (Becton & Dickinson, Meylan, France) containing a silicone separator, centrifuged within 2 hours of collection and the serum alliquoted and kept frozen at −80°C until testing. RNA was extracted from 140 μL of sera with the QIAamp viral RNA extraction kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions.

Ten microliters of the extracted RNA were tested for GBV-C/HGV RNA with a commercially available reverse transcription polymerase chain reaction kit (RT-PCR) designed to amplify the 5′UTR and NS5 regions (Boehringer Mannheim, Germany), according to the manufacturer's RT/PCR and 96-microwell detection protocol. A sample was considered to be GBV-C/HGV RNA positive when it showed a positive signal for both viral regions in a single determination, or when it was repeatedly positive only for the 5′UTR.

GBV-C/HGV RNA Quantitation

Quantitation of GBV-C/HGV RNA was carried out by real time RT-PCR using the TaqMan™ technology (Roche Molecular Diagnostics, Branchburg, NJ) in an Abiprism 7700 Sequence Detector™ (Applied Biosystems Division, Foster City, CA), which is based on the addition of a target-specific probe labeled with a reporter fluorocrome at its 5′ end and a quencher fluorescent molecule at the 3′ end. Fluorescence emission from the reporter molecule is released and detected every time the 5′ to 3′ exonuclease activity of Taq DNA polymerase breaks down the probe releasing the reporter molecule whose emission is monitored continuously during the amplification reaction. After completion of the amplification procedure, the Abiprism 7700 Sequence Detector reports the first cycle (threshold cycle) at which the fluorescence signal for each sample becomes detectable. The threshold cycle, by comparison with a standard curve generated by amplification of serial dilutions of a synthetic RNA standard of known concentration, will be proportional to the initial number of target molecules in the sample [Gibson et al., 1996; Heid et al., 1996].

For each sample, 20 μL of extracted RNA was held at 65°C for 3 minutes and kept in ice until amplification. RT/PCR was conducted in a single tube up to a volume of 50 μL containing 20 μL of RNA, 5 μL of 10× Buffer A, 10 μL of 25 M Mg^{2+} (final concentration: 5 mM), and 1.25 U of TaqGold polymerase (TaqMan PCR core reagent kit, Perkin Elmer, Branchburg, NJ), 280 μM dNTPs (PCR Nucleotide mix, Boehringer Mannheim, Germany), 25 U of M-MuLV reverse transcriptase (Boehringer Mannheim, Germany), 25 U of RNAase inhibitor (RNAse Guard, Pharmacia Biotech, Uppsala, Sweden), 4 μL of 5′UTR primers mixture (HGV primer and capture probe set, Boehringer Mannheim, Germany) (primer sense: 5′-CGG CCA AAA GGT GGT GGA TG-3′, primer antisense: 5′-CGA CGA GCC TGA CGT CGG G-3′), and 3 μL of 150 mM 5′UTR fluores-

cence-labeled probe: 5′-FAM reporter (6-carboxyfluorescein)-CCC ACC TAT AGT GGC TAC CAA GGT-TAMRA quencher (6-carboxytetramethilrhodamine). GBV-C/HGV RNA was reverse transcribed into cDNA for 30 minutes at 42°C and the cDNA amplified for 45 cycles (15 seconds at 95°C and 1 minute at 55°C).

In each run, serial ten-fold dilutions, from 10^{-3} to 10^{-7}, of a synthetic GBV-C/HGV 5′UTR transcript of known concentration were tested in parallel to generate the standard curve. The lower limit of detection of the quantitative assay, estimated from the RNA standard, was 10^3–10^4 copies/ml with a dynamic range of four logs (from 10^4 to 10^8 copies/ml). The reproducibility intra- and inter-assay was checked by testing 12 replicates of each dilution of the standard in the same run for the intra-assay, and 20 determinations for each dilution in different runs for the inter-assay. The percentage of the coefficient of variation (%CV) was calculated in both cases over the threshold cycle for each standard dilution.

NS5 Region Sequencing

Ten microliters of extracted RNA was retrotranscribed and amplified with NS5 specific primers (Forward primer: 5′-TCG AGA ATG CTG CGA GGA TT-3′; reverse primer: 5′-TGA CGG TAA GCT TTC TAG T-3′) in the following thermocycler conditions: held 30 minutes at 42°C, held 5 minutes at 95°C, and 45 cycles 15 seconds at 95°C and 1 minute at 55°C. PCR products were purified and, for each sample, a forward and reverse consensus sequence was obtained by cycle sequencing using the previously described primers (DNA Sequence kit DRhodamine terminator cycle sequencing ready reaction, Perkin Elmer, Branchburg, NJ) and automatic sequence detection (ABIPrism 310 Genetic Analyser, Applied Biosystems, Foster City, CA). The NS5 fragment analyzed spanned 297 bases from position 6786 to 7082 [Linnen et al., 1996 U45966].

GBV-C/HGV Anti-E2

Samples were tested for GBV-C/HGV anti-E2 by the pre-commercial kit μ-PLATE Anti-HGenv (Boehringer Mannheim, Germany), according to the manufacturer's instructions.

Statistical Analysis

Qualitative variables were compared using Fischer's or χ^2 tests. Quantitative variables were analyzed by Student t-test for comparison of means.

RESULTS

GBV-C/HGV distribution among patients with chronic hepatitis C was determined. One hundred nine HCV positive patients were tested for GBV-C/HGV RNA and anti-E2. The proportion of GBV-C/HGV positive markers was 21% for RNA, 36% for anti-E2, and 7% for both RNA and anti-E2. This means an overall GBV-C/HGV exposure of 64% in hepatitis C patients, much higher than the one observed in blood donors in our area [Sauleda et al., 1999].

TABLE II. GBV-C/HGV RNA Serial Quantitation Within a 6-Month Period

Index case	Number of samples tested within 6 months	$\log_{10}$GBV-C/HGV RNA MEAN ± SD
P 1	2	4.9 ± 0.02
P 2	4	5.5 ± 0.28
P 6	3	4.6 ± 0.98
P 7	4	4.9 ± 0.68
P 11	3	5.1 ± 0.52
P 12	3	6.0 ± 1.12

Serologic and Virologic Patterns in GBV-C/HGV Exposed Patients

Five anti-E2 positive patients were followed prospectively for one year with at least four serum samples. In all cases, anti-E2 antibodies maintained a constant optical density signal during the follow-up, and GBV-C/HGV viremia was not detected. The GBV-C/HGV RNA positive patients had two different patterns. Out of 31 viremic patients, 13 had a positive RT/PCR signal for the 5′ non-coding region and NS5, while the other 18 patients were repeatedly positive for 5′UTR, but negative for NS5. When this finding was compared with the anti-E2 status, none of the patients positive for both regions were anti-E2 positive, while 8 patients positive for 5′UTR and negative for NS5 were also anti-E2 positive. For these eight patients, another sample was tested after two months. In seven patients, viremia had disappeared completely while in one case it persisted with the same pattern (5′UTR positive, NS5 negative, anti-E2 positive). For the remaining ten patients who were 5′UTR positive alone (RNA NS5 negative, anti-E2 negative), eight patients could be followed up. Six cleared the viremia after two months and two patients remained 5′UTR positive NS5 negative. None seroconverted to anti-E2.

Epidemiological data including age, sex, risk factors of parenteral exposure (intra-venous drug use or transfusion), presumed age at infection, and duration of infection were analyzed in chronic hepatitis C patients. The patients were divided into three groups according to GBV-C/HGV findings: GBV-C/HGV active infection (group 1:RNA positive individuals, irrespectively of their anti-E2 status), GBV-C/HGV previously exposed patients without current infection (group 2: RNA negative, anti-E2 positive patients), and GBV-C/HGV negative patients (group 3: including patients negative for both RNA and anti-E2). Results are summarized in Table I. No significant differences were found when comparing sex, transfusion history, or age at the presumed time of infection. However, patients with active GBV-C/HGV infection were significantly younger and the estimated duration of infection was also shorter than in patients with and without past exposure to GBV-C/HGV. Patients exposed to GBV-C/HGV also reported a significantly higher frequency of intravenous drug use history than non-exposed patients did. Clini-

TABLE III. GBV-C/HGV Quantitation Before and After IFN Treatment and GBV-C/HGV and HCV Response to Treatment

Index case	Pre-IFN $\log_{10}$HGV RNA	IFN $\log_{10}$HGV RNA (BM screening)	Post-IFN $\log_{10}$HGV RNA (BM screening)	HCV response during IFN	HCV CSR after 6 months
IC 1	5.0	$<10^4$ (neg/neg)	6.7	No	No
IC 2	5.5	$<10^4$ (neg/neg)	4.3	Yes	Yes
IC 3	5.6	$<10^4$ (neg/neg)	5.5	No	No
IC 4	3.5	$<10^4$ (neg/neg)	$<10^4$ (neg/neg)	No	No
IC 6	5.7	$<10^4$ (neg/neg)	6.1	Yes	No
IC 7	4.9	$<10^4$ (neg/neg)	5.0	Yes	No
IC 9	5.2	$<10^4$ (neg/neg)	5.2	No	No
IC 10	5.6	$<10^4$ (neg/neg)	5.6	Yes	Yes

cal data regarding HCV coinfection, including ALT and γGT levels, HCV RNA quantitation, and histological score of liver biopsy, were compared between groups. No differences were found for variables related to HCV coinfection or liver damage.

GBV-C/HGV RNA Quantitation

No GBV-C/HGV RNA negative samples by screening protocol were found positive by real time RT/PCR quantitative method, thus indicating that real time quantitation is highly specific. The dynamic range of the method spans from 10^4 to 10^8 copies per mL. The standard curve of the "in vitro" GBV-C/HGV RNA transcript had a fitness, on average, of $R = 0.995$ among runs. As for the sensitivity, positive signal was detected between 10^3 and 10^4 copies per mL, which is approximately 1 $\log_{10}$ less sensitive than the screening protocol, assessed by transcript serial dilutions. Thus, it is considered that samples GBV-C/HGV RNA positive by screening RT/PCR and negative by real time PCR have less than 10^4 copies per mL. No samples with 5′UTR positive NS5 negative signal by the screening method could be quantitated as the samples fell systematically below the sensitivity threshold. The reproducibility of the method has shown to be around 1.25% CV for the intra-assay and 6% CV for the inter-assay, when considering all standard dilutions on average. The coefficient of variation of the threshold cycle increased as the concentration of the standard decreased.

Of 12 HGV/HCV coinfected patients, 11 had detectable viremia by real time quantitative RT/PCR and one had RNA titter below 10^4 copies/mL, in spite of being 5′UTR and NS5 positive for BM qualitative test. On average, they showed a viremia of 5.0 ± 0.6 $\log_{10}$ RNA copies/mL, ranging from 3.5 to 7.1 $\log_{10}$ RNA copies/mL. No differences were found when comparing mean GBV-C/HGV RNA titer of HGV/HCV coinfected patients with GBV-C/HGV RNA positive controls (5.0 ± 0.9, 3.7 to 6.1). In spite that all controls were 5′UTR and NS5 positive (and anti-E2 negative), more controls had GBV-C/HGV RNA titer lower than 10^4 copies/mL (6/21; 29%) when compared with HGV/HCV coinfected patients (2/12; 17%). However, the difference in percentage was not significant.

In six HGV/HCV coinfected patients, at least two serial samples within a period of six months could be tested (see Table II). The standard deviation of GBV-C/HGV titter ranged from 0.02 to one $\log_{10}$ GBV-C/HGV RNA copies/mL in five cases.

Analysis of GBV-C/HGV Viremia and NS5 Sequences Before And After IFN Treatment

Nine HGV/HCV patients were treated with (IFN (3MU/tiw) for 1 year due to chronic hepatitis C. They were followed-up before, during, and after treatment. One patient (IC 8) seroconverted to anti-E2 before treatment and remained GBV-C/HGV RNA negative during the follow-up. In the other eight cases, GBV-C/HGV RNA disappeared during treatment in all of them, although in seven patients GBV-C/HGV RNA was detectable again once IFN was withdrawn (Table III). Only patient IC 4, with a low pre-treatment viremia level, became a GBV-C/HGV sustained responder. ALT levels during and after treatment follow up were related to HCV response to IFN, not to GBVC-C/HGV response (Fig. 1). None of the eight patients seroconverted to anti-E2 antibodies during IFN treatment or in the post-treatment follow-up.

Pre- and post-treatment serum samples from patients IC 1, IC 2, IC 6, and IC 7 were sequenced. A fragment of 299 pb from NS5 region was amplified and consensus sequence was obtained after direct sequencing of PCR products. The consensus sequences were compared to previously described sequence (U45966) [Linnen et al., 1996]. No differences were found in pre- and post-treatment sequences in the region analyzed, except for a synonymous change in patient IC 6, where codon GAG changed to GAA. When comparing consensus sequences among patients and reference sequence, most of the changes were found in the third position of the codon. In consequence, when analyzing the aminoacid sequence, all mutations were silent, except for two changes (Asp for Glu and Lys for Glu), which maintained aminoacid chemical properties.

DISCUSSION

GBV-C/HGV markers of infection are very prevalent among HCV positive individuals, 64% of them having been exposed to the virus. Eighty-one percent of HCV positive patients with a history of intravenous drug use have been exposed to GBV-C/hepatitis G virus. We have not found GBV-C/HGV infection to be more commonly associated with a particular HCV genotype. As described previously, GBV-C/HGV does not alter pa-

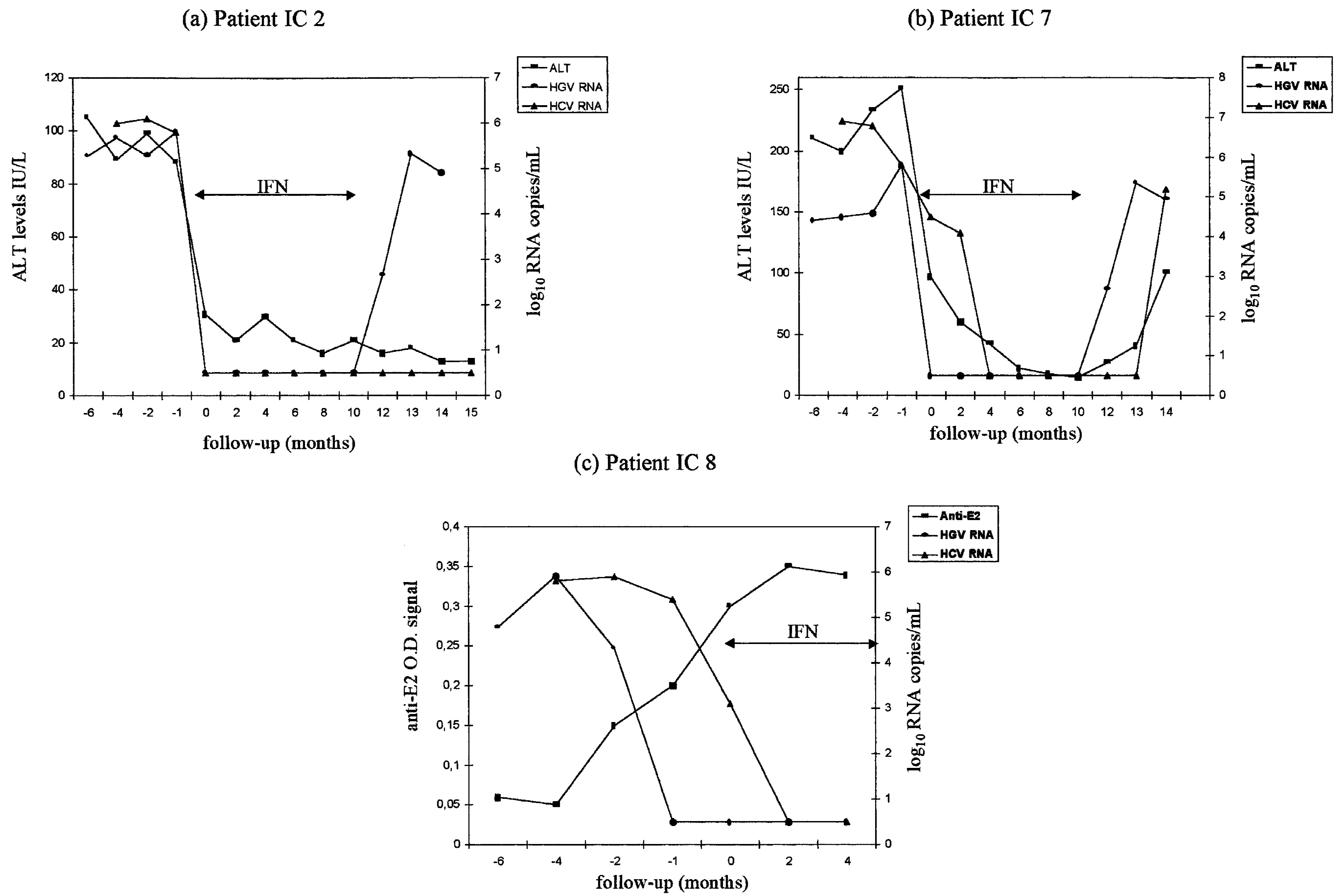

Fig. 1. GBV-C/HGV RNA, HCV RNA and ALT levels during treatment follow-up. **a:** Patient IC 2 was an GBV-C/HGV relapser but a sustained responder for HCV. ALT levels were associated with HCV response. **b:** Patient IC 7 was a relapser for both viruses. **c:** Patient IC 8 seroconverted to anti-E2 antibodies, previous to IFN treatment, coinciding with GBV-C/HGV RNA clearance from serum.

rameters of HCV infection in coinfected patients. Similarly, HCV infection does not appear to have any effects on GBV-C/HGV RNA levels, as GBV-C/HGV titer is not significantly different than that found in GBV-C/HGV positive HCV negative individuals. In HCV coinfected patients, steady GBV-C/HGV viremia was observed when serial samples over 6 months were quantitated. In contrast to published data [Martinot et al., 1997], higher mean GBV-C/HGV RNA levels were not found when compared to those of HCV. However, this finding should be interpreted cautiously since two different methods for GBV-C/HGV and HCV quantitation were used in our study (real time RT/PCR for GBV-C/HGV and Amplicor HCV Monitor™ for HCV).

Several serological patterns were noted in the natural history of GBV-C/HGV infection, which confirm and extend previous observations by other groups [Gutierrez et al., 1997; Dille et al., 1997]. Active infection is characterized by a positive RT/PCR signal for both 5′UTR and NS5 region, viremia levels over 10^4 copies/mL and by absence of detectable anti-E2 antibodies. A transition phase between active and resolved infection is characterized by RT/PCR positivity only for 5′UTR, GBV-C/HGV titers below 10^4 copies/mL and eventual presence of anti-E2 antibodies. Among patients with both GBV-C/HGV RNA and anti-E2, GBV-C/HGV viremia disappeared in the majority within 2 months. This finding confirms that simultaneous presence of both markers is a short-lived transition phase in the seroconversion process [Gutierrez et al., 1997]. Finally, antibodies to E2 are detected in resolved infections, as previously described [Tacke et al., 1997b]. Most of the patients in the study (39/70, 56%) have cleared spontaneously the infection and remained non-viremic seropositive patients. Patients with resolved infection were significantly older and the estimated interval since infection was longer than in patients with active GBV-C/HGV infection, thus indicating that spontaneous recovery is achieved with time. The fact that some patients in our study do clear spontaneously the viremia in absence of detectable antibodies to E2 might be due to delayed anti-E2 seroconversion, to lack of sensitivity of the commercial anti-E2 assay or to seroconversion to other anti-GBV-C/HGV neutralizing antibodies. In the study, all patients treated with interferon have shown GBV-C/HGV clearance during treatment. However, GBV-C/HGV infection relapsed in seven out of eight patients. The only patient in whom the antiviral treatment apparently succeeded in clearing GBV-C/HGV RNA had low pre-treatment GBV-C/HGV RNA levels (3.5 $\log_{10}$ GBV-C/HGV RNA copies/mL) and thus coincidental spontaneous clearance cannot be ruled out. However, as it is also the case with HCV infection, antiviral treatment may be more successful in patients with low GBV-C/HGV pre-treatment viremia, which could imply that IFN acts in a similar mechanism both to clear HCV and GBV-C/HGV. Similarly to HCV infection, post-treatment GBV-C/HGV RNA in relapsers returns to baseline values. Antiviral therapy does not affect GBV-C/HGV consensus sequence of NS5 fragment analyzed before and after treatment. However, it may be possible that other genome regions are changed by IFN treatment. As far as we have observed, there are no aminoacid changes within patients before and after therapy and discrete genetic variation and minimal aminoacid changes are observed among patients.

In summary, several serological patters of GBV-C/HGV infection have been observed from active highly replicating infection to complete recovery. The results lend further support to the suggestion that spontaneous clearance of infection should occur with time in a large proportion of patients. Finally, it was shown that interferon is capable of suppressing GBV-C/HGV replication and that failure to achieve a sustained response in most patients is not related to higher viral loads of GBV-C/HGV.

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